


Characterization of the Peripheral Stalk of the Vacuolar ATPase—Subunit E

An Honors Thesis (HONRS 499)

By

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ABSTRACT

V-ATPases are multisubunit ATP-dependent proton pumps consisting of two domains: a peripheral V_1 sector (subunits A-H), which binds and hydrolyses ATP, and a membrane-bound V_o sector (subunits a, c, c', c'', d, and e), which forms the pore to proton transport. V_1 and V_o subunits are held together by one central stalk made of subunits D, F, and one (or two) peripheral stalks made of C, E, G, H and the N-end of subunit a.

Subunit E (Vma 4) is a component of V_1 that forms part of the peripheral stalk connecting V_1 and V_o . Although subunit E is essential for V-ATPase assembly, its function within the complex is not known. In order to better understand the function of the peripheral stalk subunit E, site-directed mutations were performed and the analysis of these mutants is presented here.

INTRODUCTION

The vacuolar (H⁺) ATPases (V-ATPases) are multisubunit complexes found in all eukaryotic cells (1-8). V-ATPases are responsible for proton transport across intracellular membrane compartments including lysosomes, endosomes, secretory vesicles, and golgi-derived vesicles (1-8). Because V-ATPases are essential in processes like urinary acidification, bone resorption, and pH homeostasis, they are involved in diseases such as renal tubular acidosis, osteoporosis, and cancer invasiveness (1-8). V-ATPases operate by a rotary mechanism comparable to F-ATPases (F₁F₀ ATP synthases) (1-8). Because the organization of the F-ATPase is better understood they offer a useful model for comparison, however V-ATPases prove to be more complex. V-ATPases are composed of two functional domains: V₁, a peripheral domain of eight subunits (A-H) that participate in ATP hydrolysis and V_o, the integral domain responsible for unidirectional proton transport composed of six subunits (a, d, e, c, c', c'') (1-8) as seen in Table 1. The catalytic V₁ sector, responsible for ATP hydrolysis, faces the cytosolic region of the membrane and is homologous to the F₁ portion of the F-ATPase. Three A subunits (70 kDa) and three B subunits (60 kDa) participate in binding and catalytic hydrolysis of adenosine triphosphate (ATP). These two subunits are homologous to α and β subunits of F₁, subunit A binding ATP and B playing a regulatory role (1-8).

V₁V_o are attached by one central stalk and two or three peripheral stalks. The central stalk consists of subunits D and F and the peripheral stalk subunits of C, E, G, and

Table 1. V-ATPase Domains and Subunit Information

	<u>Gene (in Yeast)</u>	<u>Subunit</u>	<u>Mass</u>
V_1	VMA 1	(A)	69-kDa
	VMA 2	(B)	60-kDa
	VMA 13	(H)	54-kDa
	VMA 5	(C)	42-kDa
	VMA 8	(D)	32-kDa
	VMA 4	(E)	27-kDa
	VMA 7	(F)	14-kDa
	VMA 10	(G)	13-kDa
V_o	VPH 1/STV 1	(a)	100-kDa
	VMA 6	(d)	36-kDa
	VMA 3	(c)	17-kDa
	VMA 11	(c')	17-kDa
	VMA 16	(c'')	23-kDa
	VMA 9	(e)	10-kDa

Table 1. V-ATPases are composed of two domains. V_1 is the peripheral portion composed of eight subunits (A-H) and is responsible for the hydrolysis of ATP. V_1 is connected by central and peripheral stalks to V_o —the integral domain that provides the unidirectional proton transport across the membranes and is composed of six different subunits (a, d, c, c', c'', e).

H (5,6). ATP hydrolysis drives unidirectional proton transport involving Vo subunits a, c, c', and c''. Subunits D, a, c, and F are homologous to F-ATPase subunits γ , a, c, and e respectively (5,6). However, the remaining subunits lack clear homologs (E, F, G, H, d) with F₁Fo synthases and their roles are not yet understood.

The yeast *Saccharomyces cerevisiae* provides a model system to explore V-ATPase proton pumps (3). The subunit E, Vma4, is a 27 kDa protein of V₁ that forms part of the peripheral stalk(s) connecting V₁ and Vo and is encoded by the VMA4 gene (1-8). Subunit E can be crosslinked to subunits B, G, C, and H, suggesting that subunit E is part of the peripheral stalk (1,2,4).

Subunit E lacks homolog with the evolutionary related F-ATPase. The role of subunit E within the complex is not known and of interest in this study. To address the subunit's role in assembly and activity of V-ATPases, site-directed mutations were introduced in highly conserved residues of subunit E. As these evolutionary conserved residues may be important in the protein structure, variations in size, structure, and chemical composition may affect subunit E within the V₁Vo complex. Mutants were analyzed by examining their growth phenotype, protein stability by whole cell lysis, and assembly and activity in isolated vacuolar vesicles.

EXPERIMENTAL PROCEDURES

Site-Directed Mutagenesis

Site-directed mutagenesis of highly conserved residues of subunit E provides a method to explore the structural and functional importance of residues for subunit stability and the role of the subunit within the complex. The V-ATPase Vma4 (subunit E) protein sequence was found using available protein and gene databases online (www.yeastgenome.org). The sequence information of Vma4 in other organisms was compared in order to determine which amino acids were greatly conserved throughout evolution (Figure 1). These conserved amino acids are most likely crucial for the subunit's structural and functional stability. Mutating these amino acids within the sequence will offer some indication of the role of that residue for the subunit and V-ATPase complex. If conserved residues along the peripheral stalk subunit E are important for the structural and functional coupling of V_1 and V_o , site-directed mutations would affect V-ATPase function and/or assembly.

Point mutations at the 22nd, 24th, 27th, 28th, 32nd, 39th, and 78th amino end residues of subunit E were made, changing amino acids charge, polarity, and/or structure that could impact the tertiary structure and possibly the function of subunit E within the V-ATPase complex. In the design of the mutagenic primers, complementary oligonucleotide sequences containing the individual mutations were made. Primers contained 25 to 45 bases in length with melting temperatures of greater than or equal to 78°C, determined by the following equation:

$$T_m = 81.5 + 0.41 (\%GC) - 675/N - \% \text{ mismatch} \quad (\text{Equation 1})$$

A high percentage of glycine and cysteine (GC) enhanced the chances of proper and strong annealing due to greater amounts of hydrogen bonding than that between alanine

Figure 1. Sequence Alignment of Subunit E

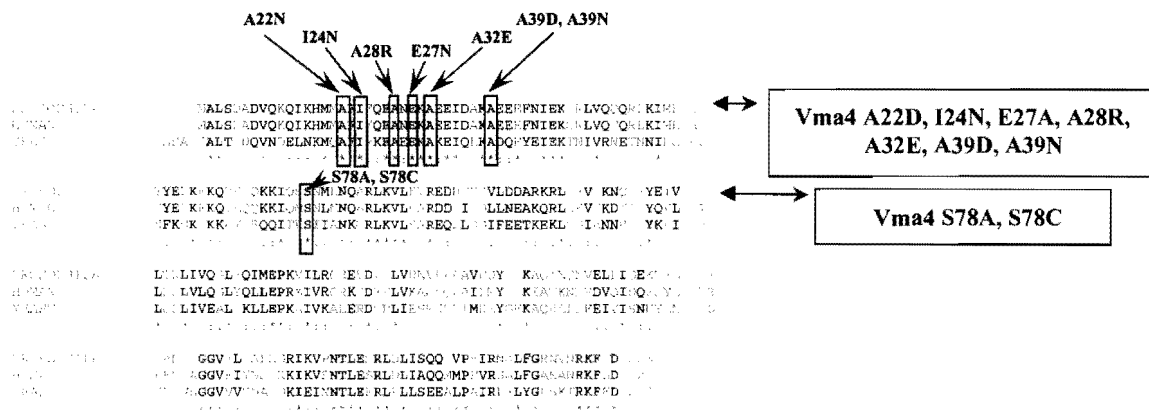


Figure. 1 Shown is the sequence alignment for subunit E from fly, human, and yeast. The blue residues correspond to fully conserved amino acids in all three species. The red residues are amino acids that have undergone conservative changes between the species. Site-directed mutations were performed on highly conserved amino acids shown by highlighted areas.

and thymine. Mutations of Vma4 were constructed using *QuikChange* Site-directed mutagenesis kit following the manufacturer's protocol. The primers for the Vma4 mutagenesis were constructed as follows with substitution sites underlined:

A22D, 5' GAACAAGATGCAAGATTTTCATCAGAAAGGAGCTGAAGAAAAAGCG 3';

I24N, 5' GAACAAGATGCAAGCTTTCAACAGAAGGAGCTGAAGAAAAAGCG 3';

A28R, 5' GCTTTCATCAGAAAGGAACGTGAAGAAAAAGCGAAGGAAATCC 3';

A32E, 5' GGAAGCTGAAGAAAAAGAGAAGGAAATCCAATTGAAGGC 3';

E37A, 5' GATGCAAGCTTTGCTTTCATCAGAAAGCAGGCTGAAGAAAAAGCG 3';

A39D, 5' GATGCAAGCTTTGCTTTCATCAGAAAGGAGGATGAAGAAAAAGCG 3';

A39N, 5' GCGAAGGAAATCCAATTCAAGAATGACCAAGAGTACG 3';

S78A, 5' GCTTTCGCAACAGATTACTAAGGCAACGATAGCAAACAAAAATG 3';

S78C, 5' GCTTTCGCAACAGATTACTAAGTGCACGATAGCAAACAAAAATG 3'

Mutations were confirmed by sequencing at Ohio State University Plant and Microbe Genomics Facility and used to transform *vma4Δ* yeast strains.

Growth Phenotype

Vacuolar Membrane ATPase (*vma*) mutants have a pH-sensitive phenotype. Wild type strains (positive control) and those mutations that do not inhibit greater than 70% of cell activity grow ideally at pH 5. At a pH higher than 7.5 and at pH 7.5 in the presence of CaCl₂ growth is inhibited. Strains in which a mutation prevents proper functioning of the V-ATPase and lose more than 70% of normal activity exhibit growth characteristics similar to the *vma4Δ* strain—a negative control lacking subunit E—because they are unable to grow at elevated pH in the presence and absence of calcium.

Mutant strains were grown in SD-Leu pH 5 media at 30°C to 0.5-1.0 OD/ml and standardized to 0.6 ODs/ml. 10-fold serial dilutions were made with ddH₂O and 2μl were

plated on pre-warmed (30°C) SD-Leu pH 5, pH 7.5, pH 7.5 + CaCl₂ media and incubated at 30°C for 24 to 72h.

Whole Cell Lysates

Whole cell lysis provides a means to determine the presence or absence of proteins within the cell. In this procedure, mutant strains were grown in SD-Leu pH 5 media at 30°C to 0.8-1.0 OD/μl. Cells were harvested, resuspended in 0.1M Tris-HCl pH 9.4 and 10mM DTT, and incubated with rocking at 30°C for five minutes. Cells were centrifuged and washed twice with 10mM Tris-HCl pH 7.5, 1.2M Sorbitol, 2% Glucose washing solution. Zymolase (10u/μl) was added to digest the cell wall during 20 minute incubation at 30°C with rocking. Cells were then washed and resuspended in 10mM Tris-HCl pH 7.5, 1.2M Sorbitol in order to remove all zymolase. The harvested pellet was combined with cracking buffer containing 5% β-mercaptoethanol and bromophenolblue at 50°C for 20 minutes until lysis was complete. Detection of V-ATPase subunits is then possible through subjection to SDS-PAGE and Western Blot techniques with primary and secondary antibodies selective for V-ATPase proteins.

Isolation of Vacuolar Membrane Vesicles

Vacuolar preparations supply pure isolated vesicles that can be analyzed for V1Vo complex assembly and activity through concanamycin-sensitive ATPase assays and Western Blot procedures, respectively. Six liters of cells were grown overnight in SD-Leu pH 5 media to a reading of approximately 1.0 OD/mL. Cells were harvested in cortex tubes at 5000 rpm, washed with 2% Glucose, and resuspended in 1.2M Sorbitol, 2% Glucose, and 10mM Tris-HCl pH 7.5. The pH was checked and if necessary, adjusted to pH 7-pH 7.5 using Tris-HCl pH 7.5. Zymolase was added (400 U/ 4000

ODs) and rocked at 150 rpm for at least an hour, until spheroplasts were identified. Spheroplasts were then centrifuged at 3500 rpm, washed with 1.2M Sorbitol, YEPD pH 5, and resuspended in Buffer A (10mM MES-Tris pH 6.9, 0.1mM MgCl₂, 12% Ficoll 400) with Aprotinin, Leupeptin, Pepstatin, and PMSF protease inhibitors. Due to the fragile nature of the spheroplasts after zymolase treatment, every step after this must be on ice. The solution is homogenized for five minutes with a chilled homogenizer and transferred to two ultracentrifuge tubes. The tubes are layered with Buffer A, balanced within 0.01 mL of each other, and spun at 24000 rpm for 35 minutes. The thin layer remaining on top after this centrifugation is collected and resuspended in 18 mL Buffer A, homogenized again for five minutes, and transferred to one tube. The solution is layered with Buffer B (10mM MES-Tris pH 6.9, 0.5mM MgCl₂, 8% Ficoll 400), balanced, and spun at 24000 rpm for 35 minutes. The final layer remaining on top after this centrifugation is collected and resuspended in MES-Tris pH 7.0, 5% glycerol. A pipette is used to homogenize the suspension and vesicles are aliquoted and stored at -80°C.

Concanamycin-A Sensitive ATPase Assay

Vacuolar vesicles are added to 1 mL cuvettes containing 25mM KCl, 25mM Tris base, 5mM MgCl₂, 0.5mM NADH, 2mM PEP, 2mM ATP, 30U/mL L-lactic dehydrogenase, and 30U/mL pyruvate kinase, pH 6.9. In this coupled enzymatic assay, a UV-Visible Spectrophotometer takes measurements every 2 seconds at 340nm, 37°C, detecting the oxidation of NADH to NAD⁺, corresponding with decreased absorbance. Changes in the level of NADH are equivalent to the amount of ATP hydrolyzed by the V-ATPase. Concanamycin-A selectively inhibits ATP hydrolysis by the V-ATPase. By

comparing hydrolysis rates of the assay with concanamycin and without concanamycin, the V-ATPase specific activity of the vesicles can be calculated. To determine the effects of a mutation on the V-ATPase activity, values obtained for each mutant were compared to the wild type.

Other Procedures

Protein Assays—Protein concentrations were determined by the Lowry Method. Bovine serum albumin was used as standard.

Western Blots— Following SDS-PAGE, proteins from the 10% gels were transferred to nitrocellulose membranes and incubated overnight with monoclonal antibodies against subunits A, B, a, and polyclonal antibodies against subunits E, d, and D. Next, membranes were incubated with alkaline phosphate conjugated secondary antibodies for two hours. The secondary antibody was then detected through color development after addition of the substrates 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT).

RESULTS

Subunit E Mutagenesis

Subunit E sequence alignment among evolutionary distant species shows high conservation of the amino acid sequence at the amino terminus (Figure 1). We hypothesized that these evolutionary conserved residues are likely important for V-ATPase function and introduced mutations within this region. Site-directed mutations were made by changing the 22nd, 28th, 32nd, and 39th alanine residues to aspartate (A22D), arginine (A28R), glutamate(A32E), and aspartate (A39D) and asparagine(A39N). The mutations changed the amino acid side chains from a nonpolar and small methyl group (alanine) to larger side chains—neutral, polar (asparagine), and charged, basic (arginine), or charged, acidic (aspartic and glutamic). Additional mutations were introduced that changed the 24th amino acid (non-polar isoleucine) to a polar asparagine (I24N). The 27th residue, glutamic acid was substituted by alanine (E27A). The 78th amino acid, a polar serine was changed to alanine as well as polar cysteine (S78A, S78C, respectively) (Table 2).

Each mutation was confirmed by sequencing, ensuring that only the desired mutation was introduced. The effect of these mutations on cell growth, subunit E stability, and V₁Vo assembly and function was examined.

Vma4 Mutants S78A and I24N Exhibit Mutant Phenotype

Cell growth characteristics of each mutant strain were compared to the wild type (positive control), and a *vma4Δ* strain carrying the vector pRS315 alone. In order to determine whether mutants exhibited *vma* phenotype, cells were grown at pH 5 but not at pH 7.5, and pH 7.5 + CaCl₂. Of the nine mutants, only S78A and I24N

Table 2. Summary of Mutations

<u>Mutation</u>	<u>Change in Chemical/Physical Property</u>
A22D	Nonpolar, small alanine ⇌ charged acidic aspartic acid
I24N	Nonpolar isoleucine ⇌ polar asparagine
E27A	Charged acidic glutamic acid ⇌ nonpolar, small alanine
A28R	Nonpolar, small alanine ⇌ charged basic arginine
A32E	Nonpolar, small alanine ⇌ charged acidic glutamatic acid
A39D	Nonpolar, small alanine ⇌ charged acidic aspartatic acid
A39N	Nonpolar, small alanine ⇌ polar asparagine
S78A	Polar –OH serine ⇌ Nonpolar, small alanine
S78C	Polar –OH serine ⇌ Polar –SH cysteine

Table 2. Summary of chemical and/or physical changes resulting from subunit E mutations.

exhibited mutant phenotype comparable to the deletion strain (Figure 2). S78A and I24N cells did not grow at pH 7.5 in the presence and absence of CaCl_2 suggesting that less than 70% of the wild type activity was retained. All the other remaining mutations showed growth comparable to the wild type.

Subunit E is Destabilized by S78A Mutation

Whole cell lysis followed by SDS-PAGE and Western blotting with antibodies against V-ATPase subunits was used to determine the stability of the subunits within the cell. Results were compared to wild type and deletion strains. The mutation S78A destabilized subunit E as lysates showed significantly lower levels of subunit E than wild type (Figure 3). All other mutations allowed expression of stable subunits, even I24N despite its mutant phenotype.

Assembly and Activity of Mutant Membranes

Vacuolar vesicles were isolated from I24N, A28R, S78A, S78C mutant strains as well as the wild-type and pRS315 strains. Vesicles were used to measure Concanamycin A-sensitive ATPase, specific activity was calculated, and the results are shown in Table 3.

Additionally, Western blot analyses of vacuolar vesicles were used to detect V_1 and V_0 subunits at the membranes. It is predicted that mutations in which subunit E is degraded or destabilized would prevent V_1 assembly and the complex would be inactive because deletion of a V_1 subunit prevents assembly of V_1 even though V_0 is assembled.

Figure. 2 Growth Phenotype

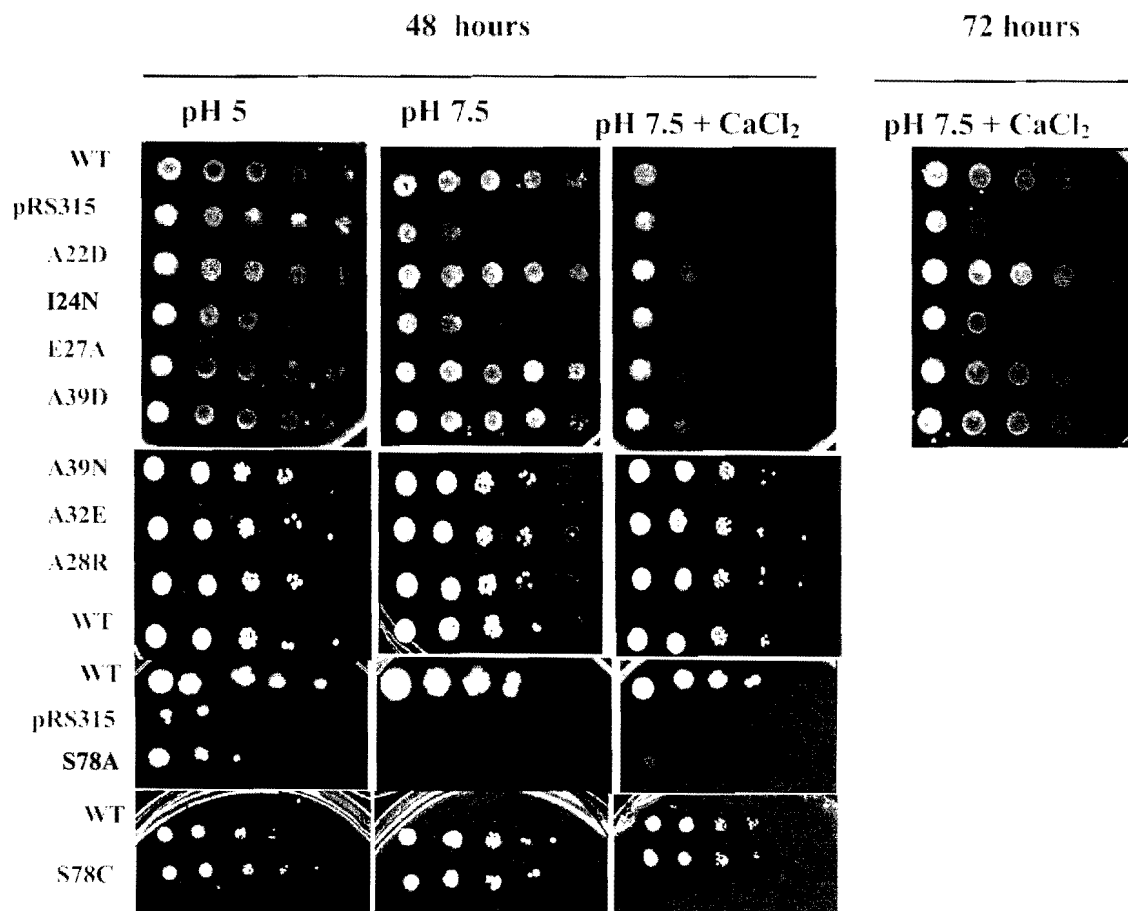


Figure. 2 Mutagenesis experiments were performed *in vitro* using the *QuikChange* Site-directed Mutagenesis kit. Mutagenized pRS315-*VMA4* was isolated and mutations confirmed by sequencing. Yeast cells lacking functional endogenous subunit E (*vma4Δ* cells) were transformed with a wild-type or mutant allele of the *VMA4* gene inserted in the CEN plasmid pRS315. Transformants were selected on synthetic minimal medium without leucine in the presence of 2% glucose (SD-Leu plates). Ten-fold dilution growth phenotype was examined at pH 5, 7.5, and pH 7.5 plus 60 mM CaCl₂.

Figure. 3 Western Blot Analysis of Whole Cell Lysates

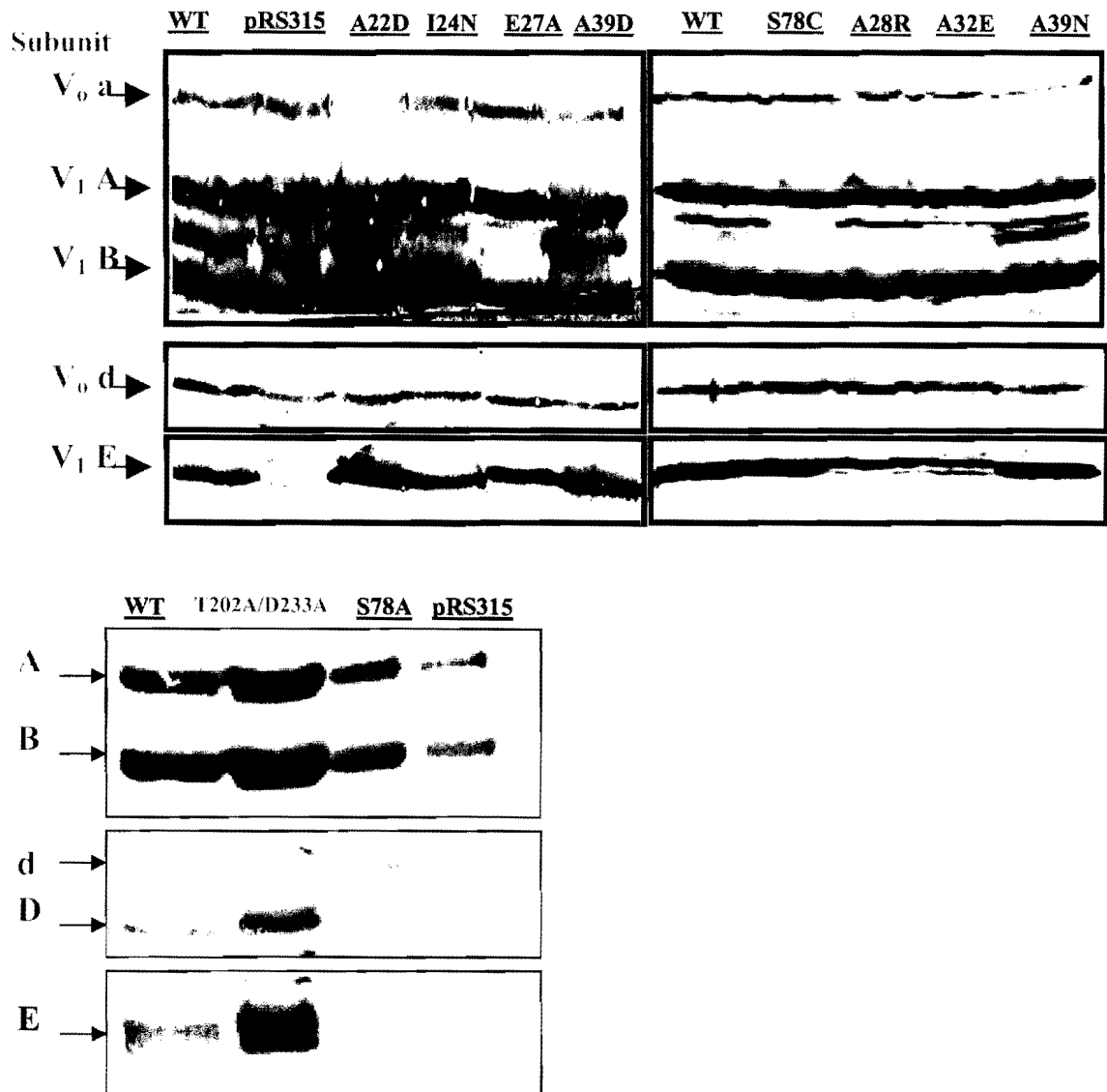


Figure. 3 Cells were converted to spheroplasts by zymolase treatment and lysed at 50°C by addition of cracking buffer containing SDS, urea, and β -mercaptoethanol. Cell lysate proteins were separated in 10% SDS-PAGE and V-ATPase subunits detected by Western Blots using antibodies against subunits a, A, B, d, D, and E. Western Blot analyses showed normal expression and stability of these subunits with the exception of the S78A, where subunit E was not detected. These results suggest that the subunit is unstable and possibly degraded due to the mutation. The presence of subunit E in all other mutant strains suggests that these mutations did not have a major effect on the stability of the subunit. The mutations described in this study are underlined in black.

Table 3. Concanamycin A Sensitive Activity in Mutant and Wild Type Membranes

<u>Yeast Strain</u>	<u>Specific Activity</u> <u>($\mu\text{molPi}/\text{min}/\text{mg}$)</u>	<u>Percentage</u>
Vma4 -WT	0.1825 ± 0.0282 (n= 7)	100 %
Vma 4Δ	$0.0142 \pm 6.5500\text{e-}3$ (n= 2)	7.8%
Vma4 -S78A	$0.0215 \pm 1.5000\text{e-}3$(n= 2)	11.7%
Vma4 -S78C	0.180 (n= 7)	95%
Vma4- I24N	Not measurable (n=3)	Not Determined
Vma4 -A28R	0.15605 (n=2)	85.5%

Table 3. Concanamycin A-sensitive ATPase activity was measured spectrophotometrically at 37°C using a coupled enzymatic assay. Protein concentrations were determined by the method of Lowry using bovine serum albumin as the standard. Concanamycin A-sensitive ATPase activity of each mutant relative to the wild-type is shown (percentage). (n)=number of vacuolar preps.

The mutations A28R and S78C retained activity showing wild type functioning and assembly (Table 3; Figure 4). The results are supported by their growth phenotype and whole cell lysis analyses that indicate mutations A28R and S78C do not alter the function of subunit E in the V-ATPase complex. Because subunit E was degraded in the S78A mutant (Figure 3), V_1 did not assemble at the membrane (Figure 4), and the enzyme was inactive (Table 3). As expected, these cells did not grow at pH 7.5 with or without CaCl_2 , showing that this mutation was detrimental to V_1V_o assembly and function, resembling the negative control.

Vesicles from the I24N strain proved to be difficult to isolate (Figure 4). The preps were scaled up in efforts to increase the yields, yet mutant membranes remained difficult to isolate and we were unable to analyze it. It is an open question why the mutation I24N was more damaging to cells than our negative control (pRS315).

Figure 4. Western Blot Analysis of Vacuolar Membranes

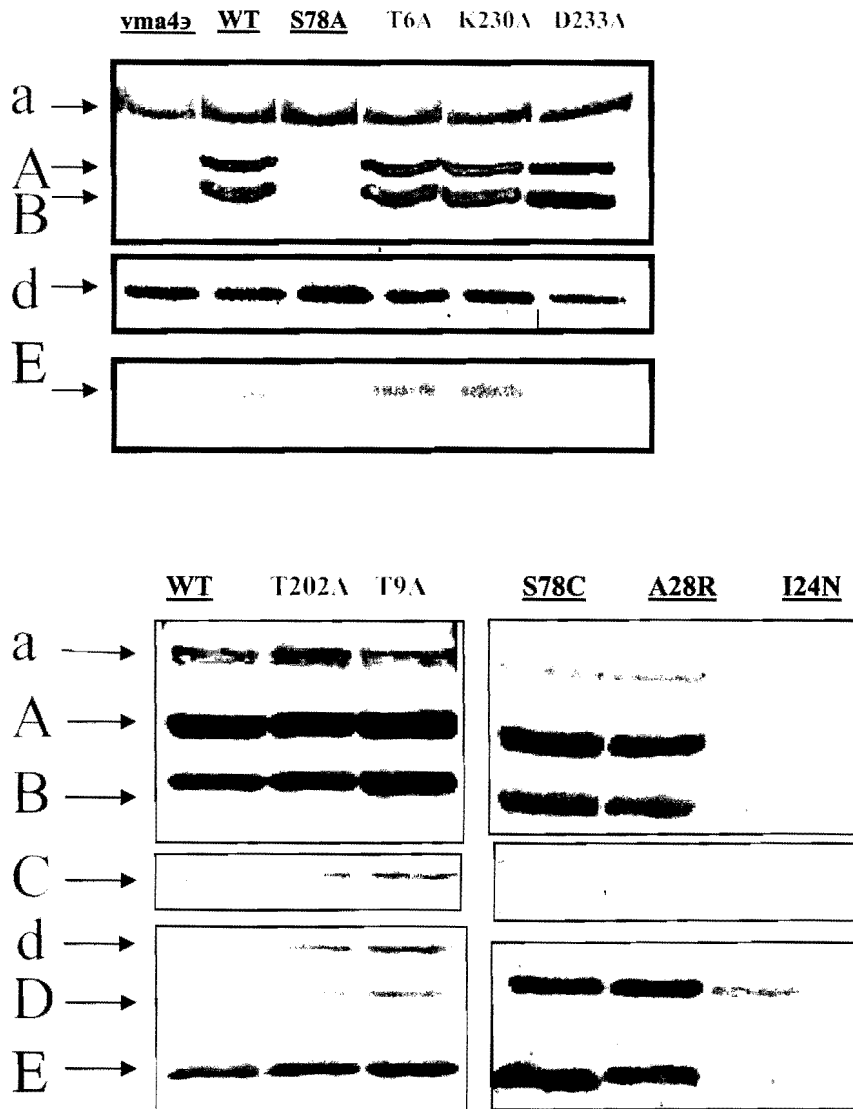


Figure 4. Cells were converted to spheroplasts by zymolase addition, lysed, and vacuoles isolated by two ficoll density gradients. Vacuolar membrane vesicles were prepared by diluting the vacuoles in a 10 mM Mes-Tris pH 6.9, 5mM MgCl₂, 5% glycerol solution. Vacuoles from wild-type and mutants strains (10 μ g) were analyzed by SDS-PAGE and Western Blots using antibodies against subunits a, A, B, C, d, D, and E. Mutants discussed in this study are shown underlined in black font.

DISCUSSION

In this study, the gene *VMA4* was mutagenized. Conserved residues within the subunit E sequence were changed to residues of different size, structure, and/or chemical properties to test the hypothesis that conserved residues along the peripheral stalk subunit E must be important for structural and functional coupling of V₁ and V_o. If our hypothesis was correct, site directed mutations would have affected V-ATPase function and/or assembly.

Site-directed mutagenesis provided valuable information about the role of subunit E within the V-ATPase complex. At the amino end, mutations at Ala-22, Iso-24, Glu-37, Ala-28, Ala-32, Ala-39, and Ser-78 were made. Growth phenotype analyses were reproducible. The mutations S78A and I24N exhibited the *vma* phenotype indicating that only 20% of the wild type V-ATPase activity or less was retained. These two mutants were the only ones that had such effect on V-ATPase function. All other mutants retained significant activity, mimicking the wild type. We concluded that S78 and I24 are residues essential for the enzyme assembly because their mutations were disadvantageous to cells. As these mutations lie within a highly conserved stretch of the subunit E sequence, they could participate in interactions with other subunits to sustain the V₁V_o complex.

Additionally, the mutant S78C had no *vma* phenotype, suggesting that replacing the –OH group with a large –SH group was compatible with V₁V_o assembly and activity.

Since subunit E was detected in I24N cells, we concluded that the *vma* phenotype was not caused by subunit E instability, rather some crucial interactions between the protein and other subunits within the V₁V_o complex were affected.

Vacuolar preparations of the wild type, pRS315, I24N, A28R, S78A, and S78C mutants provided vesicles that contained V-ATPase complexes. Isolated vacuolar vesicles provided information on V-ATPase activity and V_1V_o assembly. A28R and S78C mutant membranes had specific activities of 0.15605 and 0.180 $\mu\text{mol Pi/min/mg}$, respectively that resembled the wild type (Table 2). V_1 and V_o subunits were visualized by Western blots, indicating that V_1V_o complexes were assembled (Figure 4). These experiments indicate that the mutations did not have a major effect on the function of subunit E within the complex.

On the other hand, the specific activity of S78A was 0.0142 $\mu\text{mol Pi/min/mg}$ (Table 2). The mutation that changed Ser-78 to alanine destabilized subunit E suggesting that changing the polar residue to a nonpolar methyl group prevents important interactions involving the subunit E. Consequently, in the absence of subunit E, V_1 did not assemble and the V_1V_o complex could not be formed, leaving only assembled V_o at the membrane.

The extremely low yields of vesicles carrying mutation I24N did not allow measurement of ATP hydrolysis and their Western blots analysis showed no proteins. Further studies need to address why I24N had such a drastic effect on the vacuolar preparation. In addition, vacuolar preparations need to be performed for the remaining mutant strains A22D, A32E, E37A, A39D, and A39N. Although these mutants lack mutant phenotype, and whole cell lyses results suggest that these mutations did not destabilize subunit E and allowed for significant V_1V_o assembly and activity, vacuolar membranes have to be studied and their specific activity determined.

In conclusion, the mutation S78A was unique because it caused degradation or destabilization of subunit E, preventing the V-ATPase from functioning properly. I24N was particularly interesting because only traces of membranes could be isolated. These results suggest that subunit E interactions within the complex are crucial for the V-ATPase to operate effectively. That S78C resembled wild type in assembly and activity, suggests that the polar interactions at Ser-78 are necessary for function and stability. Although still inconclusive, the I24N mutant will offer some interesting insights about the need of a nonpolar residue at this position.

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